

Nuclear markers confirm taxonomic status and relationships among highly endangered and closely related right whale species

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Right whales (genus: *Eubalaena*) are among the most endangered mammals, yet their taxonomy and phylogeny have been questioned. A phylogenetic hypothesis based on mitochondrial DNA (mtDNA) variation recently prompted a taxonomic revision, increasing the number of right whale species to three. We critically evaluated this hypothesis using sequence data from 13 nuclear DNA (nuDNA) loci as well as the mtDNA control region. Fixed diagnostic characters among the nuclear markers strongly support the hypothesis of three genetically distinct species, despite the lack of any diagnostic morphological characters. A phylogenetic analysis of all data produced a strict consensus cladogram with strong support at nodes that define each right whale species as well as relationships among species. Results showed very little conflict among the individual partitions as well as congruence between the mtDNA and nuDNA datasets. These data clearly demonstrate the strength of using numerous independent genetic markers during a phylogenetic analysis of closely related species. In evaluating phylogenetic support contributed by individual loci, 11 of the 14 loci provided support for at least one of the nodes of interest to this study. Only a single marker (mtDNA control region) provided support at all four nodes. A study using any single nuclear marker would have failed to support the proposed phylogeny, and a strong phylogenetic hypothesis was only revealed by the simultaneous analysis of many nuclear loci. In addition, nuDNA and mtDNA data provided complementary levels of support at nodes of different evolutionary depth indicating that the combined use of mtDNA and nuDNA data is both practical and desirable.

Keywords: right whales; endangered cetaceans; conservation; phylogeny; taxonomy; nuclear DNA

1. INTRODUCTION

The delimitation of species boundaries is a fundamental aspect within evolutionary biology and often provides the focus for many biological conservation efforts (but see entire proceedings devoted to these topics: Godfray 2004; Goldstein *et al.* 2000; MacCallum 2001). Typically, character-based or tree-based methods are used to operationally delimit species boundaries under criteria presented within the framework of a variety of species concepts (Sites & Marshall 2003). Establishing taxonomic boundaries and reconstructing phylogenies of closely related cetaceans has been hindered by a lack of diagnostic morphological characters. Recently, two new cetacean species have been proposed through the use of genetic data (see special issues: Dalebout *et al.* 2002; Wada *et al.* 2003). In addition new taxonomic boundaries have recently been accepted for populations of minke whales, beaked whales and right whales (Bakke *et al.* 1996; Rosenbaum *et al.* 2000; Dalebout 2003). These discoveries mirror a wider

trend in the systematics of marine taxa to use genetic data for identification of cryptic species (Knowlton 1993; Hualkasin *et al.* 2003; Rawson *et al.* 2003).

Cryptic species lack diagnostic morphological characters (McGovern & Hellberg 2003) owing to evolutionary morphostasis or failure to identify any phenotypic differences (Knowlton 1993; Dalebout 2003). Among certain taxa, preservation of soft tissue is difficult, leading to the loss or destruction of morphological characters (Knowlton 1993). In the case of cetaceans, the ability to measure morphological characters or to sample a sufficient number of individuals can be difficult compared with smaller organisms. Also, field measurements are hindered by the minimal time cetaceans spend at the water's surface.

Molecular genetic data can help define species boundaries (Vogler & DeSalle 1994; Palumbi 1996). Mitochondrial DNA (mtDNA) sequences are the most commonly used genetic data for analysing fine population structure and species relationships in mammals (Moritz *et al.* 1987; Dizon *et al.* 1997). The popularity of mtDNA is in a large part owing to its rapid rate of sequence divergence (Gissi *et al.* 2000), availability of universal primers (Kocher *et al.* 1989; Sorenson *et al.* 1999), and the ability to compare datasets across taxa (Johns & Avise 1998). These attractive

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features, and the theoretical expectation that genetic drift will often be stronger for mtDNA compared with nuclear DNA (nuDNA) loci, arguably make mtDNA the preferred marker for initial descriptions of molecular diversity (Moore 1995). However, several biological factors can cause single gene phylogenies to be incongruent with the species tree, as has been demonstrated in a diverse range of species (Pamilo & Nei 1988; Avise 1989; Sota & Vogler 2001; Ballard *et al.* 2002). Any gene tree can differ from the species phylogeny owing to random lineage sorting (Pamilo & Nei 1988), motivating a consensus approach applied across independent loci (Hare 2001). Unfortunately, it is impossible to sample independent genealogical histories within the mitochondrial genome because it is inherited as a single unit and recombination of heteroplasmic forms is rare (Rokas *et al.* 2003). In addition, mtDNA gene trees, even when strongly supported, may be particularly likely to deviate from the species tree owing to maternal inheritance (Evans *et al.* 2003), selection (Ballard *et al.* 2002), introgression (Shaw 2002) or unrecognized sampling of paralogous gene copies (Thalmann *et al.* 2004).

Nuclear loci can provide independent estimates of phylogenetic relationships to corroborate or refute phylogenies constructed using mtDNA sequence data. While any one nuclear locus can also suffer from the complications cited above, cumulatively they can provide more reliable results than phylogenies based on a single genetic marker (Wu 1991; Cao *et al.* 1994; Brower & DeSalle 1998).

Introns are a common source of nuclear sequence data because they evolve with fewer evolutionary constraints relative to coding sequence (Palumbi & Baker 1994; Prychitko & Moore 1997; Friesen *et al.* 1999; Hare & Palumbi 2003): polymerase chain reaction (PCR) primers for amplifying nuclear introns in mammals have become readily available (Lyons *et al.* 1997).

Management strategies designed for the protection of endangered wildlife often hinge on an established taxonomy and hypothesized set of relationships among closely related organisms (e.g. Avise & Nelson 1989; Baillie & Groombridge 1996). Questions of taxonomy have serious implications for conservation and management, and these issues have been the subject of intense debate among the cetacean conservation community (Reeves *et al.* 2003). For right whales (genus: *Eubalaena*), a group that includes some of the most endangered and well-studied populations of large whales (Best *et al.* 2001), the number of species and hypothesized phylogenetic relationships were recently revised based solely on mtDNA results (Rosenbaum *et al.* 2000; Best *et al.* 2001). Right whales include 10 historically recognized populations, organized into three groups, including North Pacific, North Atlantic and Southern Hemisphere right whales, the latter encompassing all Southern Hemisphere populations. Traditionally, North Pacific and North Atlantic right whales were identified as a single species *Eubalaena glacialis*, to the exclusion of southern right whales, *Eubalaena australis* (Muller 1954; Ivanova 1961; Brownell *et al.* 1986).

The recent phylogenetic analysis based on mtDNA control region sequences revised the classification of right whales into three separate species (Rosenbaum *et al.* 2000). That study was the first phylogenetic survey to incorporate

right whale samples from all three groups, and supported the presence of three distinct maternal clades. As a result, the International Whaling Commission and the United States Government recently chose to recognize North Pacific right whales as a distinct species, *Eubalaena japonica* (Best *et al.* 2001). Recent acoustical recordings (Mellinger *et al.* 2004) and sightings of North Pacific right whales in areas previously devoid of right whales, emphasize the importance of proper classification of these critically endangered whales. No previous morphological characteristics have distinguished three species of right whales; however, many studies have not adequately examined the appropriate characters. A recent study by Cooper (2004) suggests that some morphological differentiation based on character data does exist that distinguishes the three right whale species. All right whales were afforded protection in 1935 and each of the right whale species is currently listed as either endangered or vulnerable by the International Union for the Conservation of Nature and Natural Resources (Reeves & Leatherwood 1994; Reeves *et al.* 2003). However, hunting of whales by commercial whaling vessels continued into the 1970s (Yablokov 1994; Brownell *et al.* 2001; LeDuc *et al.* 2001). The rapid incorporation of mtDNA results into government policy suggests that their impact on management strategies is potentially large. This, in part, provided motivation to our efforts, to critically test the phylogenetic hypothesis generated from the mtDNA data.

The objectives of this study were to determine the number of species supported by a combined analysis of 13 nuclear markers and to determine the phylogenetic relationships among right whales utilizing both nuclear and mitochondrial markers.

2. MATERIALS AND METHODS

(a) *Samples*

In order to examine the pattern of variation in the nuclear genome, samples used in this study represent all three recognized species of right whales, *E. glacialis* ($n=8$), *E. australis* ($n=11$) and *E. japonica* ($n=6$). In an effort to maximize the amount of variation found among nuclear loci, samples were included that represent different localities and unique mtDNA lineages revealed in Rosenbaum *et al.* (2000). A single sample representing *Balaena mysticetus* was included as an out-group specimen. All samples were collected by biopsy sampling of free-ranging animals or from stranded animals.

(b) *Molecular methods*

All DNA was extracted from skin biopsies using either a standard phenol–chloroform method (Sambrook *et al.* 1989) or Qiagen DNA Easy Tissue Extraction Kit (QIAGEN). Primers used for PCR amplification are listed in table 1. All regions were amplified using the PCR (Saiki *et al.* 1988) with the following parameters: 95 °C (2 min) preliminary denaturing, 35 cycles of 95 °C (15 s) denaturing, 47–64 °C (10–30 s) annealing and 72 °C (1–2 min) extension, followed by a single cycle of 72 °C (4 min). Annealing temperatures used for each of the sets of primers can be found in table 1. Primers were first tested using published protocols (if available, e.g. Lyons *et al.* 1997) followed by adjustment of annealing temperature or MgCl concentration to optimize PCR performance if necessary.

Table 1. Nuclear and mitochondrial loci used in the present study.

loci (abbreviation used in text)	size of PCR product	primer source	optimum annealing temperature
actin (ACT) ^a	1413	Palumbi and Baker (1994) ^b	60
biglycan mRNA (BGN) ^a	680	Lyons <i>et al.</i> (1997)	60
alpha-lactalbumin (LAC) ^a	628	Milinkovitch <i>et al.</i> (1998)	57
mas oncogene (MAS)	734	Vrana <i>et al.</i> (2000)	58
oxytocin (OXY) ^a	374	P. B. Vrana (personal communication)	52
parathyroid hormone mRNA (PTH)	310	Lyons <i>et al.</i> (1997)	52
calmodulin-dependent kinase (CAMK) ^a	2173	Lyons <i>et al.</i> (1997) ^b	64
cadherin-2 mRNA (CDH2) ^a	2137	Lyons <i>et al.</i> (1997) ^b	57.5
ceruloplasmin mRNA (CP) ^a	1263	Lyons <i>et al.</i> (1997) ^b	60–64
esterase D (ESD) ^a	770	Lyons <i>et al.</i> (1997) ^b	56
fibrinogen gamma-polypeptide (FGG) ^a	1122	Lyons <i>et al.</i> (1997)	55
beta-hexosaminidase (HEX) ^a	2171	Lyons <i>et al.</i> (1997)	60
proteolipid protein (PLP) ^a	1057	Lyons <i>et al.</i> (1997) ^b	60
mitochondrial DNA (control region; mtDNA)	292	Rosenbaum <i>et al.</i> (1997) ^b	47

^a Sequence spans one or more introns.

^b Internal primers used during sequencing.

The PCR products were cycle-sequenced with dye-labelled terminators using conditions recommended by the manufacturer (Applied Biosystems). Sequence reactions were analysed using an ABI-Prism model 377, 3100 or 3700 Genetic Analyser (PE Applied Biosystem, Foster City, CA). In most cases, PCR products were sequenced directly using PCR primers. However, for five loci with long amplicons, PCR products from some individuals were cloned and three clones per individual were sequenced to infer a majority-rule haplotype free of *taq* misincorporations. For eight loci (table 1), internal primers were designed to increase yield of PCR product and for sequencing (primer sequences available from the authors). Sequences were aligned using SEQUENCHER 4.1 software. A single exception was a large insertion/deletion found in the PLP intron, which was aligned using CLUSTALW (Thompson *et al.* 1994).

So as to determine the extent of variability within each group, shorter gene regions that produced phylogenetically informative characters were screened across all samples. Once taxonomic boundaries were determined, longer introns, CAMK, CDH2, CP, ESD, FGG and HEX were screened in a subset of samples (*E. glacialis* ($n=1$), *E. australis* ($n=2$) and *E. japonica* ($n=2$)) for the purpose of phylogenetic analysis. The combined dataset incorporated the mtDNA control region sequence data from Rosenbaum *et al.* (2000).

(c) Population aggregation analysis

Population aggregation analysis (PAA) (Davis & Nixon 1992) is a framework to empirically diagnose phylogenetic species. In our study we used samples representing different mtDNA lineages in right whales. The PAA was performed according to the diagnostic character framework of Davis & Nixon (1992). In PAA, operational taxonomic units are defined by the presence of fixed nucleotide characters, which define a group (species) to the exclusion of other groups, as opposed to traits, which are shared among populations and are indicative of population-level frequency differences.

(d) Phylogenetic analyses

Phylogenetic analyses were performed for the combined dataset using all loci, as well as the combined nuclear dataset,

using PAUP* v. 4.0b10 (Swofford 2002). All characters were weighted equally with the exception of three insertion/deletions (indels) detected that were greater than a single base pair. One indel was found in PLP and two were detected in HEX. Each indel was scored as present or absent (1 or 0, respectively) in the final analysis. Maximum parsimony analyses were conducted using heuristic searches with stepwise addition trees, replicated 1000 times. Tree bisection-reconnection (TBR) was chosen as the branch-swapping algorithm. Five samples (North Atlantic, $n=4$; southern, $n=1$) were removed from the analysis, as they were identified as 'taxonomic equivalents' (Wilkinson 1995), showing identical character states to other samples. The omission of these samples improved the resolution of the phylogenetic analysis by reducing the number of equally parsimonious trees from greater than 200 000 to 99. Heterozygous sites were verified through cloning and generation of multiple sequences. Samples not screened across all loci were coded as missing data for those markers.

(e) Partition homogeneity test

The partition homogeneity test (PHT) was used to test for congruence between the nuclear and mitochondrial datasets (Farris *et al.* 1994). In the PHT, heuristic searches were performed with simple taxon addition and TBR branch swapping, and replicated 100 times using PAUP* v. 4.0b10 (Swofford 2002).

(f) Measure of nodal support

The amount of support for individual nodes was evaluated by calculating bootstrap values (BP) through random character sampling (Felsenstein 1985). The BP were generated in PAUP* from 1000 replicates of 100 addition-heuristic searches and TBR branch swapping.

Branch support (BS) (Bremer 1988, 1994) was calculated as positive, negative and zero values as per Gatesy *et al.* (1999b). The BS measures the number of extra steps in tree length that is required to produce a tree without a particular node. Partitioned branch support (PBS) values (Baker & DeSalle 1997) were calculated in order to summarize the amount of support or conflict at a particular node on the

Table 2. Partitioned branch support (PBS) and population aggregation analysis (PAA) values obtained for the simultaneous analysis of all datasets. (Values represent the contribution of each locus (and the combined nuclear dataset) to the branch support (BS) partitioned across the four nodes supporting species boundaries or sister-taxa relationships on the strict consensus tree (nodes 1–4, [figure 1a](#)). In addition, the number of phylogenetically informative (PI) characters are given for each locus after excluding the out-group.)

locus	size of PCR product (bp)	PI	North Atlantic (node 1)		southern (node 2)		North Pacific (node 3)		Southern + North Pacific sister-clade (node 4) PBS
			PAA	PBS	PAA	PBS	PAA	PBS	
CAMK	2173	19	9	0.15	2	0	6	0	0
HEX	2171	12	5	1	4	1	3	0	0
CDH2	2137	3	0	−0.15	0	0	0	0	0
ACT	1413	9	1	0.09	2	0	1	0.09	3
CP	1263	8	0	0.15	1	0	1	0	0
FGG	1122	5	2	0	2	0	0	0	0
PLP	1057	12	0	−0.17	0	−1	0	1.72	−2
ESD	770	2	0	0	0	0	1	0	0
MAS	734	3	0	1	0	−1	1	1.05	0
BGN	680	1	0	0	0	0	0	0	0
LAC	628	4	2	2	0	0	0	0	0
OXY	374	2	0	0	0	0	0	0	0
PTH	310	4	0	1	3	2	0	0	0
nuDNA	14 832	84	19	5.07	14	1	13	2.89	1
mtDNA	292	32	4	2.93	3	6	3	11.1	3
Total	15 124	116	23	8	17	7	16	15	4

phylogenetic tree, contributed by individual loci as well as the nuclear dataset. PBS is used as an indicator of node-specific support contributed by data partitions. Searches were performed in PAUP* v. 4.0b10 ([Swofford 2002](#)) as described ([Baker & DeSalle 1997](#)). Minimum length constrained topologies were derived from heuristic searches with 100 random addition replicates and TBR branch swapping.

Dataset influence (DI) ([Gatesy, *et al.* 1999b](#)) was measured to better understand which markers exhibit positive influence over the phylogenetic analysis through the removal of individual partitions from the combined dataset. The DI calculates the difference in BS of a dataset with or without a particular partition. Analogous to PBS, DI is an indication of the amount of signal contributed by an individual data partition. However, DI differs from PBS by analysing support for the entire tree as opposed to individual nodes and through the revelation of hidden support that emerges with the incorporation of the partition ([Gatesy *et al.* 1999a](#)). Support for a particular topology may be hidden by PBS analysis owing to conflicting signal. A partition with a negative or zero PBS value may contain data that support the overall topology, and in this respect contain hidden support. The DI scores were divided by the minimum possible number of steps for informative characters in the dataset so that datasets of different sizes could be compared (scaled DI; [Gatesy, *et al.* 1999b](#)).

3. RESULTS

(a) Population aggregation analysis

The PAA ([table 2](#)) revealed that North Atlantic right whales possess 19 diagnostic nucleotide characters among the nuclear loci. These were combined with the four characteristic sites of the mtDNA yielding a total of 23 diagnostic characters that unambiguously provide a

diagnostic framework for North Atlantic right whales. The number of diagnostic nucleotide characters for North Pacific right whales increases from the reported 3 in mtDNA to 16 with the inclusion of the nuclear loci. In addition to the 3 mtDNA nucleotide positions that diagnose southern right whales from North Atlantic and North Pacific right whales, we report here an additional 14 diagnostic characters. Based on coalescence theory, a random sample of six genes from each of two populations provides better than 95% confidence in a result showing fixed differences. That is, the diagnostic characters are likely to be robust to further sampling and are unlikely to appear in a panmictic population ([Hey 1991](#)).

(b) Phylogenetic analysis

In an attempt to address the evolutionary history of three closely related cetacean species, the phylogenetic analyses presented here incorporated a dataset of 13 nuclear markers spanning over 14 kb of sequence. These data were combined with the mtDNA control region data presented in [Rosenbaum *et al.* \(2000\)](#). The PHT of the mitochondrial control region versus the combined nuclear dataset revealed the datasets were significantly incongruent separate (and $p = 0.01$).

Among the nuclear partitions, 84 nucleotide positions were determined to be informative within a parsimony framework (synapomorphs). The amount of phylogenetic signal found in each marker varied considerably ([table 2](#)) with a range of 1 (BGN) to 19 (CAMK) parsimony informative characters. A parsimony analysis of the combined nuclear dataset produced a strict consensus of 5912 equally parsimonious trees (score = 103; [figure 1b](#)). The mtDNA dataset included 32 additional parsimony informative nucleotide positions. A strict consensus of 99

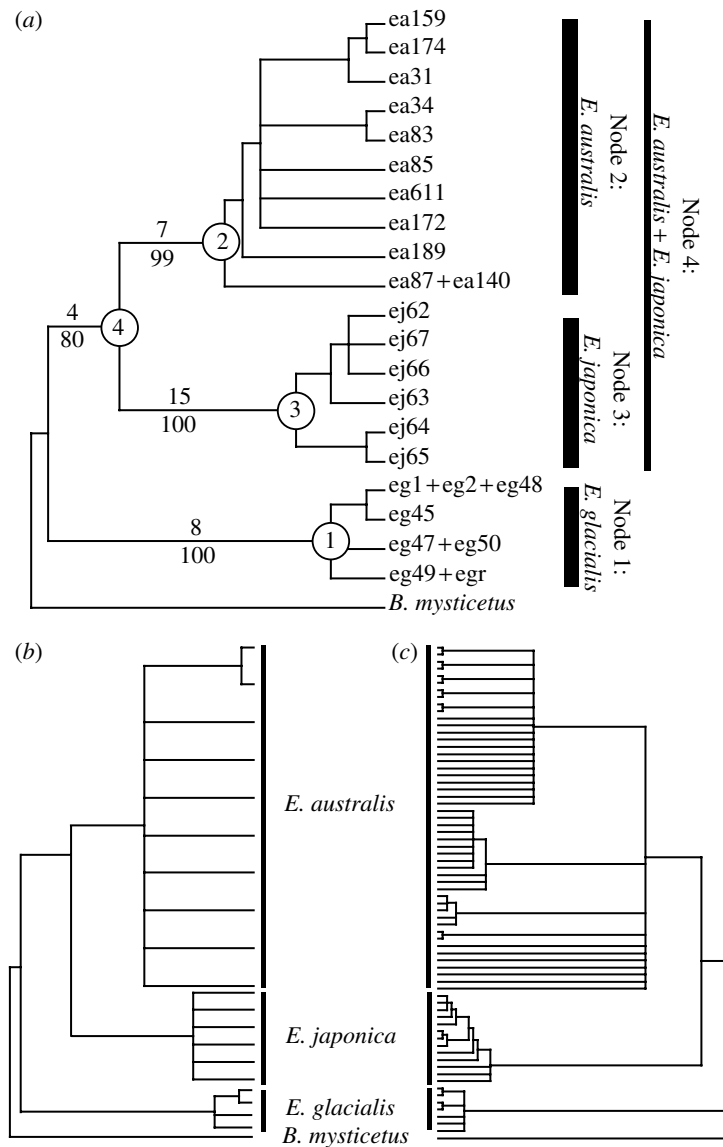


Figure 1. (a) The strict consensus cladogram obtained from the simultaneous analysis of 13 nuclear loci and the mitochondrial DNA (mtDNA) control region for *Eubalaena*. The total evidence tree is a consensus of 99 most parsimonious trees (Length = 167, CI = 0.73, RI = 0.84). Circled numbers on branches indicate the four nodes that define each *Eubalaena* species as well as relationships among species (1 = *E. glacialis*, 2 = *E. australis*, 3 = *E. japonica*, 4 = *E. australis* and *E. japonica* sister-taxa relationship). Support for each of these nodes is represented by branch support values above the branch and bootstrap values below each branch. (b) The strict consensus cladogram obtained from the combined analysis of 13 nuclear loci. (c) The strict consensus cladogram obtained from the phylogenetic analysis of the mtDNA control region (Rosenbaum *et al.* 2000).

equally parsimonious trees (score = 167) was produced representing the combined analysis of 13 nuclear gene regions and the mtDNA D-loop region (figure 1a).

The phylogenetic analyses described above resulted in monophyly of right whales relative to *B. mysticetus* and resolved three right whale clades of interest. Each right whale species forms a well-defined monophyletic clade supported by high BP values and positive BS values (figure 1a, nodes 1–3). These nodes correspond to North Atlantic right whales (node 1; BS = 8, BP = 100), southern right whales (node 2; BS = 7, BP = 99) and North Pacific right whales (node 3; BS = 14, BP = 100). In addition, the combined analysis produced a well-supported sister-taxa relationship between North Pacific and southern right whales (figure 1a, node 4; BS = 4, BP = 80).

(c) Measures of nodal support

The BS values for the nodes that define each right whale group (nodes 1–3) as well as the North Pacific and southern right whale sister group relationship (node 4) are indicated in figure 1a. Each of the nodes of interest receives a positive BS score in the combined analysis. PBS values were calculated to determine the relative amount of BS contributed by the mtDNA control region as well as the combined nuDNA dataset (table 2). Both the mtDNA and the nuDNA datasets contribute positive PBS to all four nodes. Furthermore, the PBS, for nuDNA, data was further broken down to ascertain the amount of support or conflict contributed by each of the individual nuclear loci. All nodes were characterized by either support (PBS > 0) or lack of conflict (PBS = 0) contributed by nuclear partitions and the mtDNA control region. Negative PBS

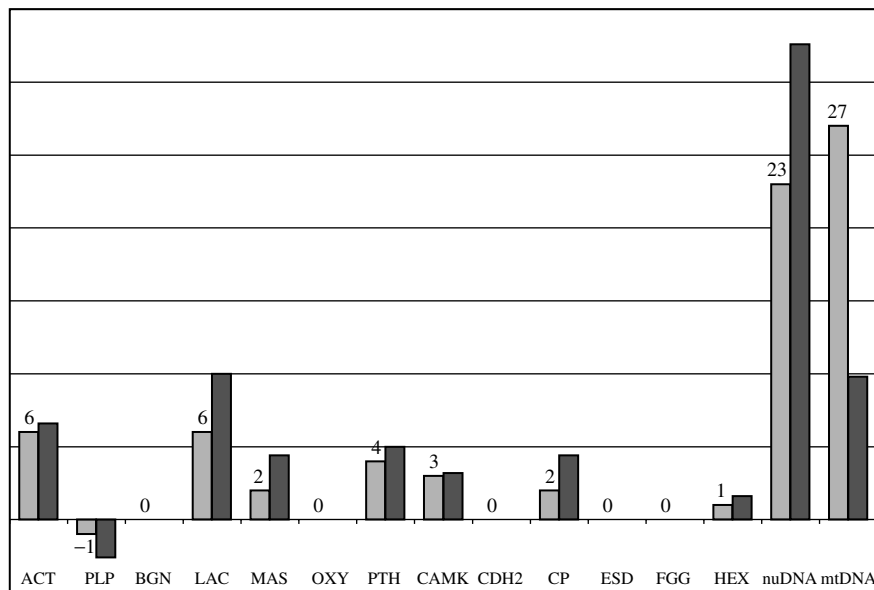


Figure 2. Dataset influence (DI; light grey) and scaled dataset influence (SDI; dark grey) values for each nuclear loci as well as the mitochondrial DNA control region and the combined dataset of 13 nuclear loci (a summation of individual locus values). The DI values are found above respective columns and heights of SDI columns are relative. For SDI values see text.

values, when they occurred for a locus, were small and found at only one of the four nodes tested. The exception to this is a single marker, PLP, where three of the four nodes had negative PBS as extreme as -2 .

(d) Dataset influence

Figure 2 summarizes DI values for each partition. According to these results, the 15 datasets have the following order of influence in a combined analysis: mtDNA (+27), ACT (+6), LAC (+6), PTH (+4), CAMK (+3), MAS (+2), CP (+2), HEX (+1), BGN (0), OXY (0), CDH2 (0), ESD (0), FGG (0), PLP (-1). DI can also be scaled to the minimum number of character steps for informative characters in a particular dataset (scaled dataset influence (SDI); Gatesy, *et al.* 1999b) so that datasets of different sizes can be compared. When the DI values are scaled (figure 2), the order of overall influence changes to: LAC (0.50), mtDNA (0.49), ACT (0.33), PTH (0.25), MAS (0.22), CP (0.22), CAMK (0.16), HEX (0.08), BGN (0), OXY (0), CDH2 (0), ESD (0), FGG (0), PLP (-0.13).

4. DISCUSSION

(a) Utility of nuclear loci

It has been suggested that nuclear markers would not be useful to elucidate relationships among closely related species owing to the slower rate of evolution of the nuclear genome (Moore 1995; DeBry & Seshadri 2001; Palumbi *et al.* 2001). However, it has been shown that in certain cases, nuclear markers can be used to corroborate or refute phylogenetic relationships derived through mtDNA analyses (Baker *et al.* 2001; Macnish *et al.* 2002; Shaw 2002; Slowinski & Lawson 2002). Several studies have shown that multiple locus datasets can produce a biologically reasonable phylogeny from a simultaneous analysis, whereas contradictory phylogenies result from analysis of individual markers (Baker & DeSalle 1997;

Gatesy *et al.* 1999a,b; O'Grady 1999). At the very least, inclusion of numerous nuclear loci can provide a stronger analysis of genetic structure and can increase the likelihood of deriving the true species tree by incorporating biparentally inherited markers and sampling across independent evolutionary loci (Baker & DeSalle 1997; Gatesy, *et al.* 1999b; Palumbi *et al.* 2001; Russello & Amato 2003). Data from additional species groups are needed to empirically determine how often phylogenies of closely related species can be resolved through the combined analysis of nuDNA sequences from multiple loci.

Our study clearly reveals the utility of nuclear markers in a phylogenetic study of closely related species with little morphological differentiation, while demonstrating that this strength comes only from utilizing multiple loci. PHT revealed that the nuDNA and mtDNA datasets were statistically incongruent. This result was not unexpected as the most parsimonious trees of the mtDNA (figure 1c; Rosenbaum *et al.* 2000) and the combined nuclear dataset (figure 1b) included numerous shallow intraspecific nodes that differed. Intraspecific incongruence is expected among loci unless there is strong population structure within species (Baum & Shaw 1995). We further explored congruence by using PBS to measure the relative support contributed by each dataset to the nodes defining ocean-specific and higher order clades. Both the mtDNA and nuDNA datasets showed positive BS for nodes defining each right whale species as well as the node supporting the southern right whale and North Pacific right whale sister-taxa relationship. These results indicate that the mtDNA and nuDNA datasets are congruent with respect to nodes bearing on species identity and relationships.

We further partitioned BS to compare individual nuDNA loci and found varying levels of support at the different nodes of interest. Parsimony analysis of individual partitions failed to produce phylogenetic resolution for most nuclear markers used in this study (data not shown). None of the nuclear markers provided positive PBS values

at all four of the nodes of interest (table 2). However, eight nuclear markers provided positive PBS for at least one of the nodes of interest, and in only five instances did a nuclear marker conflict with one of the nodes ($PBS < 0$). Only a single nuclear marker, PLP, provided negative PBS at more than one node. Despite these differences in information among individual markers, the additive effects of incorporating 13 nuclear markers in a combined analysis leads to significant resolution of the four nodes that define all three right whale species and strong overall support for previously hypothesized relationships among the different right whale species.

The DI values reveal that seven of the nuclear markers have a positive influence ($DI > 0$; figure 2) and five have neither support nor conflict ($DI = 0$; figure 2) on the resolved tree. A single nuclear marker (PLP; figure 2) provides a negative DI value, suggesting that only PLP is incongruent with the overall analysis. Comparison of PBS and SDI values indicate different levels of support are provided by the mtDNA and nuDNA datasets. Summation of PBS values for the mtDNA dataset across all four nodes of interest (23.03; table 2) is considerably greater than that of the combined nuDNA dataset (9.96; table 2). However, SDI values summed across the nuclear loci (1.63; figure 2) are considerably greater than SDI from mtDNA data (0.49; figure 2). This indicates that a considerable amount of hidden support is found among the nuclear markers. This hidden support is only revealed during the simultaneous analysis of all loci.

Disagreement among the nuclear markers could be owing to several factors, including hybridization between lineages, homoplasy and chance events of lineage sorting during speciation events (Avise 1994; Moore 1995; Maddison 1997). Homoplasy was minimal with the 13 nuclear loci as indicated by high CI (consistency index) and RI (retention index) values (figure 1a) but cannot be discounted even between closely related species (O'Uigin *et al.* 2002). Hybridization is unlikely owing to the anti-tropical distribution of right whales and their offset breeding seasons (Davies 1963; Rosenbaum *et al.* 2000). Retention of ancestral polymorphisms is a plausible explanation for incongruence among loci in right whales because the internode branch length was relatively short between North Atlantic right whales and the subsequent divergence of southern and North Pacific right whales (Davies 1963; Slade *et al.* 1994; Moore 1995; Rosenbaum *et al.* 2000; Broughton & Harrison 2003). The one exception to this generalization might be the PLP locus because it showed discordance at nearly every node, suggesting a more systematic cause for the departure.

Overall, our results with nuclear loci showed weak and largely concordant phylogenetic signals from individual markers. Strong phylogenetic resolution only emerged during the simultaneous analysis of multiple loci (Gatesy *et al.* 1999b; Sota & Vogler 2001). The inclusion of numerous independent markers helped reveal evolutionary relationships that were not discerned through the analysis of individual nuDNA markers (Gatesy *et al.* 1999b; O'Grady 1999).

In this study, despite conflicting signal from one marker (PLP) and a lack of overall influence exhibited by five of the nuclear loci (figure 2), a highly resolved phylogenetic

tree was produced during the simultaneous analysis of 14 independent markers. Equivalent data from any one nuclear locus probably would not have recovered as much phylogenetic signal across all nodes of interest. Any such gene tree would again suffer from the potential stochastic and sampling errors inherent in single locus phylogenies. In addition, the use of both a mitochondrial marker and numerous nuclear loci added strength to this phylogenetic study owing to complementary resolving power (Johnson & Sorenson 1998; Johnson & Clayton 2000). This is apparent in our study, as the PBS values for the combined nuclear dataset and the mtDNA dataset differed considerably at the nodes of interest (table 2). For example, PBS for the most ancestral clade, North Atlantic right whales, was higher from nuDNA markers than from mtDNA. Thus, instead of partitioning the theoretical strengths and weaknesses of nuDNA relative to mtDNA (Moore 1995), it appears that their combined use is both practical as well as desirable.

(b) Implications for right whale species relationships

The PAA survey of nuclear markers for right whales clearly demonstrates the presence of diagnostic molecular characters for each of the three species. These characters are consistent with an absence of gene flow for a significant period of time to allow fixation of these characters owing to genetic drift or natural selection, despite the potential for inter-oceanic dispersal. These diagnostic characters could allow for accurate identification of any right whale sample to ocean of origin.

While data from our analysis reflect a deep genetic divergence and an extended period of genetic isolation, this evolution appears to have occurred with little or no obvious morphological differentiation. The presence of a strong phylogenetic signal among closely related species has two potential explanations. The first is that the rate of divergence in these nuDNA markers is greater than would have been expected based on mtDNA (Palumbi *et al.* 2001). The second explanation is that the three species of right whales are more divergent than is suggested by morphology. Among other mysticete cetaceans, most species exhibit genetic structuring at the population level across oceans (Baker *et al.* 1993; Berube *et al.* 1998), but only minke whales show genetic fixation across ocean basins (Baker *et al.* 2000). Like right whales, sequence data indicates that minke whales have genetically isolated populations in the North Atlantic, the North Pacific and the Southern Hemisphere, although the ancestor-descendant polarity has not been established (Baker *et al.* 2000).

Under a character-based approach, the molecular data presented here clearly support the delimitation of three right whale species (Davis & Nixon 1992; Goldstein *et al.* 2000; Wiens & Servedio 2000). Templeton (1994) argued that in addition to an absence of gene flow, empirically demonstrated through diagnostic characters, a phylogenetic species must meet two further criteria. The first is that the groups should be phylogenetically distinct (Templeton 1994). The character-based approach and subsequent parsimony analysis produced from this dataset supports the presence of three phylogenetic species (figure 1a), as well as a strong sister-taxon relationship

between North Pacific and southern right whales (figure 1a, node 4). This clearly refutes the traditional classification of a single 'northern' right whale species, as the North Pacific and southern right whale are more closely related to each other than either is to the North Atlantic group. The second criterion is that each group should demonstrate ecological or demographic non-exchangeability (Templeton 1994). Distributional data of right whales from each of the three ocean basins shows that the three groups show no range overlap and that the populations are most probably allopatric (Best *et al.* 2001). These classifications can be used to define all three species as ecologically significant units (Vogler & DeSalle 1994).

Proponents of the biological species concept (BSC) typically favour a more traditional and conservative classification of a single right whale species with various numbers of sub-species. However, these contentions are based on a lack of evidence as opposed to evidence in support of reproductive compatibility (i.e. Baker *et al.* 2003). The nuclear data presented in this study, in conjunction with the mtDNA dataset of Rosenbaum *et al.* (2000) and the biogeographical data of right whales strongly supports reproductive isolation and three distinct species, despite a lack of any documented diagnostic morphological differences among the three groups. While testing the BSC is impossible with allopatric right whale populations, mechanisms promoting reproductive isolation include anti-tropical behaviour, the formation of the Panamanian isthmus, and long glacial periods in the Arctic.

(c) Conclusion

We present our combined data phylogeny of right whale relationships and the taxonomic status of individual species within *Eubalaena*. The congruence between the tree based on the 13 nuclear markers and the mtDNA gene tree, as well as the consensus tree from the combined dataset, strongly supports the notion that the true species tree was recovered from the combined analysis. Given the level of differentiation detected between closely related species of right whales, our approach and these nuclear loci may have additional utility for examining relationships among cetaceans and other closely related species where taxonomy and systematics have been unresolved.

Notwithstanding the strong support for taxonomic revision provided by the robust mtDNA analysis (Rosenbaum *et al.* 2000), the results here, based on 14 presumably independent markers, provide a stronger genomic justification for the recognition of a new species, *E. japonica*, in the North Pacific. The importance of this taxonomic revision is underscored by the recent increase in sightings of *E. japonica* that have significantly expanded the number of known individuals (LeDuc *et al.* 2001). These observations combined with acoustic studies (Mellinger *et al.* 2004) and our current genetic findings strongly support additional conservation management measures for this critically endangered species.

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